SELF-IMMOBILIZING BIOCATALYSTS FOR FLUIDIC REACTION CASCADES

Patrick Bitterwolf[1], Theo Peschke[1], Manfred Maier[1], Sabrina Gallus[1], Teresa Burgahn[1], Esther Mittmann[1], Martin Peng[1], Kersten S. Rabe[1], Christof M. Niemeyer[1]

[1]Institute for Biological Interfaces (IBG 1), Karlsruhe Institute of Technology, Karlsruhe, Germany

ABSTRACT

The industrial implementation of whole-cells and enzymes in flow biocatalysis microreactors is essential for the emergence of a biobased circular economy. Major challenges concern the efficient immobilization of delicate enzymes inside miniaturized reactors without compromising their catalytic activity. We describe the design and application of four different immobilization techniques including self-immobilizing whole-cells and purified enzymes on magnetic microbeads, as well as reactor modules manufactured by 3D printing of bioinks containing thermostable enzymes. To increase the volumetric activity of our microreactors we furthermore developed and applied self-assembling all-enzyme hydrogels with cofactor-regenerating capabilities. The resulting reactor formats have excellent operational stability times of > 14 days and maximum space-time yields of > 450 g product/$L^{-1}day^{-1}$ paving the way for mild and effective immobilization techniques of biocatalysts in microfluidic systems.

Keywords: self-immobilizing, enzymes, continuous flow biocatalysis, cascade reactions, stereoselective

INTRODUCTION

Biocatalysis is currently being transformed by the use of biomimetic fluidic approaches enabling compartmentalized multiple enzyme cascades for the sustainable and continuous production of fine- and value-added chemicals in the industrial "white" biotechnology sector.[1, 2] Apart from optimizing the biocatalysts by protein engineering, effective immobilization techniques are essential to enable precise temporal and spatial reaction control in fluidic microreactors. Thus for the establishment of artificial fluidic cascades new approaches for the immobilization of accessible and active biocatalysts are needed.

RESEARCH CONCEPT

In order to retain the active biocatalysts under continuous flow conditions, we developed different "self-immobilizing" biocatalysts including from whole-cells[3] or pure enzymes[4] in combination with superparamagnetic microparticles as well as reactor modules manufactured by 3D printing of bioinks containing thermostable enzymes.[5] To further increase the volumetric activity of our microreactors, we developed self-assembling and cofactor-regenerating all-enzyme hydrogels, consisting of either an (*R*)- or an (*S*)-enantiospecific ketoreductase and the nicotinamide adenine dinucleotide phosphate regenerating glucose 1-dehydrogenase.[6, 7]

RESULTS & DISCUSSION

For the design of "self-immobilizing" whole-cells we generated bacterial strains that can selectively bind to solid substrates containing an appropriate binding tag,. In an initial work, we employed the cell-surface display of the strepatividin-binding peptide (SBP), the SpyTag/SpyCatcher (ST/SC) system or a HaloTag variant based on the outer membrane protein Lpp-ompA , which led to a spontaneous covalent coupling of magnetic microbeads carrying streptavidin (STV), a ST/SC component or a chlorohexyl (CH) -moiety.[3] At the same time functional content could be expressed in the cytosol of the immobilized cells, as we demonstrated

https://doi.org/10.24355/dbbs.084-202001220953-0

employing fluorescent proteins or stereoselective ketoreductase enzymes. The latter strains gave high selectivities for specific immobilization onto complementary surfaces and also in the whole-cell stereospecific transformation of a prochiral C_S-symmetric nitrodiketone.

Implementing enzyme cascades into future biocatalytic processes enables the use of compartmentalized microfluidic reactors. However, a major challenge in the establishment of microfluidic enzyme cascades concerns the immobilization of isolated enzymes. To this end we developed "self-immobilizing" enzyme biocatalysts based on (*R*)- and (*S*)-selective ketoreductases in combination with an NADPH regenerating glucose-1-dehydrogenase anchored on magnetic microbeads.[4] The enzymes were tagged with SBP, ST or halo-based oligonucleotide binder (HOB), respectively, and bound to the corresponding streptavidin (STV), SC or chlorohexyl (CH) coated microbeads. The enzyme-modified beads were loaded in four-channel microfluidic chips to create compartments that have the capability for either (*R*)- or (*S*)-selective reduction of the prochiral C_S-symmetrical substrate 5-nitrononane-2,8-dione (NDK). Analysis of the isomeric hydroxyketone and diol products by chiral HPLC was used to quantitatively characterize the performance of reactors configured with different amounts of the enzymes. Long operating times of up to 14 days indicated stable enzyme immobilization and the general robustness of the reactor. Even more important, by fine-tuning of compartment size and loading, the overall product distribution could be controlled to selectively produce a single meso diol with nearly quantitative conversion (>95%) and excellent stereoselectivity (d.r. > 99:1) in a continuous flow process.

In a parallel approach we demonstrate the utility of thermostable enzymes in the generation of biocatalytic agarose-based inks for a simple temperature-controlled 3D printing process.[5] As examples we utilized an esterase and an alcohol dehydrogenase from thermophilic organisms as well as a decarboxylase that was thermostabilized by directed protein evolution. We used the resulting 3D-printed parts for a continuous, two-step sequential biotransformation in a fluidic setup.

To further increase the volumetric activity of such reactors, we have recently developed self-assembling all-enzyme hydrogels with cofactor-regenerating capabilities, consisting 100% of either an (*R*)- or an (*S*)-enantiospecific ketoreductase combined with the NADPH-regenerating glucose 1-dehydrogenase.[6] Mounted in microfluidic reactors, the gels show excellent stereoselectivity (> 99% ee.) with near quantitative conversion (> 90%) in the reduction of different prochiral ketones along with high robustness under process and storage conditions. The gels constitute a compartment in which reaction intermediates are retained, thereby enabling extraordinary high total turnover numbers of the expensive cofactor NADP(H). Coupling of such reactor modules allows to facilitate multi step reaction cascades.

CONCLUSIONS

The field of biocatalysis demands practical and effective methods for the immobilization of the corresponding biocatalysts. We developed four different approaches including whole-cells, purified enzymes as well as enzymes bound or entrapped into carrier materials like magnetic microbeads or agarose, respectively.

Maximizing space–time yields (STY) of biocatalytic flow processes is essential for the establishment of a circular biobased economy. We present a comparative study in which different biocatalytic flow reactor concepts were tested with the same enzyme, the (*R*)-selective alcohol dehydrogenase from *Lactobacillus brevis* (LbADH).[7] To enable cross-platform comparison, STY values were determined for the various reactor modules. While mono- and multilayer coatings of the reactor surface led to STY < 10, higher productivity was achieved with packed-bed reactors (STY ≈ 100) and the densely packed hydrogels (STY > 450). The latter modules could be operated for prolonged times (>6 days). Given that our approach is not limited to specific enzymes, we anticipate that compartmentalized microfluidic reaction modules equipped with self-immobilizing biocatalysts would be of great utility for numerous biocatalytic and even chemo-enzymatic cascade reactions under continuous flow conditions.

https://doi.org/10.24355/dbbs.084-202001220953-0

ACKNOWLEDGMENT

This work was supported by the Helmholtz program BioInterfaces in Technology and Medicine and Deutsche Forschungsgemeinschaft. We thank Alessandro Angelin, Maximillian Grösche, Jens Bauer, Anke Dech, Ruben Garrecht, Marc Skoupi, Ann-Kathrin Schneider, Cornelia Ziegler for experimental help and Ishtiaq Ahmed for the synthesis of the nitrodiketone. SG & PB are grateful for a Kekulé fellowship by Fonds der Chemischen Industrie.

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https://doi.org/10.24355/dbbs.084-202001220953-0